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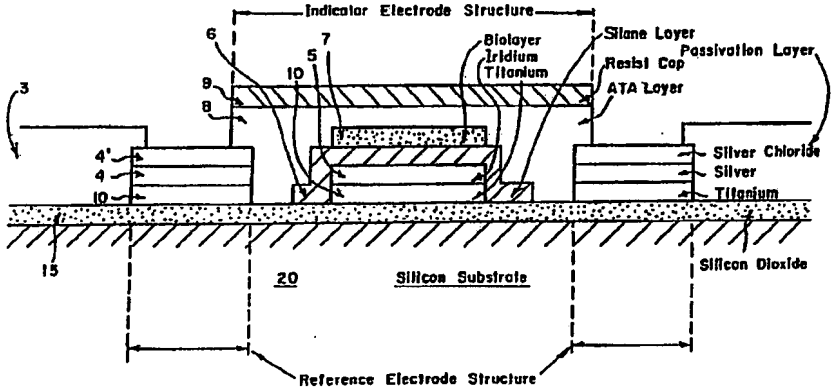
(57) Abstract

An efficient method for the microfabrication of electronic devices which have been adapted for the analyses of biologically significant analyte species is described. The techniques of the present invention allow for close control over the dimensional features of the various components and layers established on a suitable substrate. Such control extends to those parts of the devices which incorporate the biological components which enable these devices to function as biological sensors. The materials and methods disclosed herein thus provide an effective means for the mass production of uniform wholly microfabricated biosensors. Various embodiments of the devices themselves are described herein which are especially suited for real time analyses of biological samples in a clinical setting. In particular, the present invention describes assays which can be performed using certain ligand/ligand receptor-based biosensor embodiments. The present invention also discloses a novel method for the electrochemical detection of particular analyte species of biological and physiological significance using a substrate/label signal generating pair which produces a change in the concentration of electroactive species selected from the group consisting of dioxygen and hydrogen peroxyde.



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WHOLLY MICROFABRICATED BIOSENSORS AND PROCESS FOR THE MANUFACTURE AND USE THEREOF

1. FIELD OF THE INVENTION

This invention relates to wholly microfabricated biosensors, methods and materials for the mass production thereof, and their use in the determination of the presence and/or concentration of a variety of selected analyte species. In particular, the integrated biosensors of the present invention may be manufactured by a process which allows the incorporation of a variety of bioactive molecules, which bioactive molecules provide the basis of the analytical technique, through the use of materials which are compatible with the bioactive molecules and which materials have been especially adapted for that purpose. The integrated biosensors of the instant invention are also fully compatible with undiluted biological fluids and may be utilized in a wide range of medical, as well as nonmedical, applications.

More particularly, this invention relates to novel electrochemical assay procedures and to novel wholly microfabricated biosensors useful in determining the presence and/or concentration of biological species (analytes) of interest. The invention also relates to the novel use of a non-electroactive substrate (hereinafter the "substrate") that does not undergo detectable oxidation or reduction at an electrode's operating potential, but which substrate undergoes a reaction with a substrate converter which gives rise to changes in the concentration of detectable electroactive species, these changes are measured and related to the concentration of the biological species of interest. Additionally, the invention pertains to methods for the microfabrication of the biosensor.

The assay procedures and biosensor of this invention are also exemplified as being useful in effecting immunoassays. Such immunoassays are also exemplified wherein

the substrate converter is an enzyme (alkaline phosphatase) that reacts with the substrate (5-bromo-4-chloro-3-indoxyl phosphate) to produce changes in the concentration of electroactive species (dioxygen and hydrogen peroxide) which are electrochemically detected with the biosensor, an immunosensor in this instance. Both sandwich and competitive assays can be effected using the procedures and biosensor of the present invention. In these assays, one embodiment of the biosensor comprises a base sensor comprising a catalytic electrode and optional reference electrode, an adhesion promoter layer overlaid on the biosensor, and a bioactive layer that is covalently immobilized on the adhesion promoter layer, which bioactive layer is a receptor of the immunological analyte of interest.

2. BACKGROUND OF THE INVENTION

Great effort has been expended in the development of chemical sensors which can measure the presence and/or concentration of chemical species in blood or other biological fluids. These sensors can be macroelectrodes (nonmicrofabricated) of the everyday bench top variety for measuring the pH of samples, and they may sometimes take the form of microelectrodes suitable for implantation within the body of a subject. Such devices are presently made individually or in certain cases by a combination of hand assembly and manufacturing methods which may include the thin-film and photoresist techniques currently used to manufacture integrated circuits (See, for example, Pace, S., Sensors and Actuators 1982, 1, 475; Zemel, J.N., United States Patent No. 4,302,530 in which is disclosed a method for fabricating a "substance-sensitive" photodefinable layer over semiconductor devices, especially ion-selective field effect transistors (ISFET)). In spite of this considerable and continuous effort, sensors based upon this ISFET technology have not become common articles of commerce. The

fact is that wholly microfabricated biosensors, that is, sensors which are uniformly mass produced solely by thin-film techniques and the micromanufacturing methods, useful in the clinical setting and adaptable to the detection and measurement of a whole host of chemical and biological species, have not been manufactured successfully.

It is apparent that the degree of complexity involved with the mass production of commercially viable biosensors is much more formidable than even those persons of ordinary skill in the art once perceived. Of major concern is the compatibility of inherently harsh physical and chemical processes, associated with existing semiconductor manufacturing methods, with sensitive organic compounds and labile biologically active molecules which comprise part of a functioning biological sensor. An article by Eleccion (Eleccion, M. Electronics 1986, June 2, 26-30) describes the current state of affairs with regard to microsensors and makes brief references to active areas of research including the detection of specific ions, gases, and biological materials. Progress in the area of field effect transistors (FETs) is noted and problems and limitations with present manufacturing methods are discussed.

Numerous other review articles describe a variety of electrochemical devices including ion-selective electrodes (ISEs) and ISFETs which incorporate enzymes or immunoactive species (See, for example, Pinkerton, T.C. and Lawson, B.L. Clin. Chem. 1982, 28(9), 1946-1955; Lowe, C.R. Trends in Biotech. 1984, 2(3), 59-65; Koryta, J. Electrochim. Acta 1986, 31(5), 515-520; DeYoung, H.G. High Tech. 1983, Nov., 41-50; Davis, G. Biosensors 1986, 2, 101-124 and references cited therein). Also, the general principles of operation of enzyme-based sensors have been reviewed (See, Carr, P.W. and Bowers, L.D. Immobilized Enzymes in Analytical and Clinical Chemistry, Wiley-Interscience (1980). Various mathematical models of operation have been examined, including the

external mass-transfer model by Racine, P. and Mindt, W. Experientia Suppl. 1971, 18, 525. Significant problems and limitations in the fabrication of these devices remain unconquered, however, especially with regard to the fabrication of sensors intended for the analysis of nonionic species. The mass production of biosensors based upon ion-selective electrodes (ISEs) would be particularly useful as these sensors can be adapted easily for the analysis both of ionic as well as uncharged analyte species.

It is also important to note that in current clinical settings medical practitioners commonly request that analyses of one or more components of a complex biological fluid such as whole blood. Currently, such analyses require a certain amount of processing of the whole blood, such as filtration and centrifugation, to avoid contamination of the instruments or to simplify subsequent measurements. Frequently, blood samples are sent to a remote central facility where the analyses are performed. Patients are thus deprived of valuable information which, in most cases, is not available for hours, sometimes days. Clearly, substantial advantages can be envisaged if analyses on undiluted samples can be carried out and if instruments or sensors can be produced which can perform real-time measurements.

2.1. REPRESENTATIVE NONMICROFABRICATED ELECTRODES

It should be pointed out that many glucose sensors have been constructed using nonmicrofabricated or "macro" electrodes (See, for example, Fischer, U. and Abel, P., Transactions of the American Society of Artificial Internal Organs 1982, 28, 245-248; Rehwald, W., Pflugers Archiv 1984, 400, 348-402; Gough, D.A., United States Patent No. 4,484,987; Abe, H. et al., United States Patent No. 4,515,584; Lunkska, E., United States Patent No. 4,679,562; and Skelly, P., UK Patent Application 2,194,843). However,

no aspect of thin-film processing is described in the manufacturing processes disclosed by the references cited above.

The combination of a layer containing the enzyme urease and an ammonium ion-selective electrode or an ammonia gas sensing electrode is known in the art. A recent example of such a diagnostic system is described by Conover, G., et al. in U.S. Patent No. 4,713,165. In this system, a nitrocellulose membrane is immersed in a solution of the enzyme urease which is absorbed into the membrane. This enzyme-containing membrane, in its dessicated state, is then mounted onto the surface of an ammonium ISE. The resulting macroelectrode device is used to perform a blood urea nitrogen (BUN) measurement in biological fluids, such as serum, plasma, blood, and the like.

Another illustrative example of the earlier approaches to the manufacture of urea sensors is described by Williams in U.S. Patent No. 3,776,819. Similar to the previous reference, a urease layer is coated over a cation-sensitive electrode, which layer may comprise urease and gelatin, fibrin, or filter paper pulp. An outer semipermeable membrane made from collodion (a cellulosic material) or cellophane is common, also.

2.2. PREVIOUS ATTEMPTS AT MASS PRODUCTION

While the unit cell of a base sensor, typically an electrode, can be duplicated on a planar surface such as a silicon wafer (See, Bergveld, P., IEEE Transactions of Biomedical Engineering BME 1972, 19, 342-51), a viable method for the deposition of a complex set of layers which confers selectivity and sensitivity to the base sensor has not been demonstrated or shown to be fully compatible with reported integrated circuit processing techniques. Such complex layers would contain relatively labile biological molecules such as ionophores, enzymes, antibodies, antigens or

fragments thereof and are, in general, weak and sensitive to mechanical agitation. Although such layers may be applied onto a wafer, preventing their inactivation and/or destruction due to further processing steps is not readily achieved because such processing commonly includes exposing the wafer to organic chemicals, strong acids and bases, heat, or subjecting the wafer to mechanical agitation, dicing, or scribing, usually accompanied by wash steps which employ low-pressure water-jets.

To prevent the destruction of these fragile layers, it has been a common practice in the prior art to dice or cut the semiconductor wafer into individual base sensors before the biolayers are established. Any additional packaging (e.g., wire bonding the sensor to a connector, encapsulating the device to provide adequate passivation) is also performed prior to applying the biologically active layers. Such complete devices are, therefore, produced only partially in a manner which is compatible with automated mass production methods. For example, the enzyme urease has been deposited onto the gate of a single pre-encapsulated ion-selective field effect transistor (ISFET) (Karube, I. et al., Analytica Chimica Acta 1986, 185, 195-200).

European Patent Application No. 0 012 035 provides ample discussion regarding the deficiencies of current FET devices, foremost of which is their limited applicability to the analysis of nonionic species. In an attempt to combine electrochemistry and semiconductor technology, miniaturized multiple sensors are fabricated on a single chip. The utility of this reference is limited, however, because the disclosure only speaks in general terms and contains no enabling description of the critical biolayers and protective barriers which are critical to the successful microfabrication of functional biosensors. In fact, only materials such as cellulose and a poly(vinyl chloride) (PVC) layer containing valinomycin (sensitive to potassium ions) or

nonactin (sensitive to ammonium ions) are specifically disclosed, and the deficiencies of these materials have been known in the biosensor art for sometime. Representative articles on the subject of PVC membranes and the like for use in ISEs abound and include: Davies, D.G. et al. Analyst 1988, 113, 497-500; Morf, W.E. Studies in Analytical Chemistry, Punger, E. et al. (Eds.), Elsevier, Amsterdam (1981) p. 264; Ammann, D. Ion-Selective Microelectrodes, Springer (1986); Oesch, U. et al. Clin. Chem. 1986, 32. 1448; Oggenfuss, P. et al. Analytica Chim. Acta 1986, 180, 299; Thomas J.D.R. Ibid. 1986, 289; and Thomas, J.D.R. J. Chem. Soc. Faraday Trans. I 1986, 82, 1135.

Also, certain Japanese publications merit some discussion. Japanese Application No. 61-234349 describes a FET semiconductor biosensor coated with a solution of enzyme and a crosslinking agent to provide a crosslinked layer over the entire semiconductor. Separate applications of commonly used photoresist materials are then required to protect desired areas from a subsequent treatment of protease. Reliance on enzymatic digestion of undesired protein layers is expected to give unreliable and unsuitable dimensional control. Precise dimensional control is an important consideration in the mass production of microstructures. Japanese Application No. 61-283862 discloses a procedure for fixing an enzyme membrane by applying a polymer solution containing an enzyme on a solid surface, drying, applying a crosslinking agent to the resulting film through a mask, and removing noncrosslinked portions of said film. Again, such a technique fails to take advantage of standard photoresist technology and can only lead to a poorly resolved pattern. Another reference, Japanese Application No. 61-254845 employs the typical approach of immersing sensor elements in enzyme-containing solutions and then selectively inactivating the membranes.

2.2.1. PHOTOPATTERNING METHODS

The use of photosensitive synthetic polymers to provide patterned membranes is known. For instance, glucose oxidase has been mixed with a photosensitive synthetic polymer mixture consisting of poly(vinyl pyrrolidone) (PVP) and 2,5-bis(4'-azo-2'-sulfobenzal)cyclopentanone (BASC) (See, Hanazato, Y. et al. Anal. Chim. Acta 1987, 193, 87; Hanazato, Y. et al. in European Patent Application No. 0 228 259). The resulting mixture was then used to establish a patterned membrane on a single ISFET device. Equal parts of glucose oxidase and bovine serum albumin (BSA) were used in the mixture which was irradiated and developed using aqueous 1-3% glutaraldehyde. In this system, in which the matrix is a synthetic photosensitive polymer, the authors discuss a number of unsolved problems including saturation of the sensor response at concentrations of glucose above about 3 mM and poor long term stability probably caused by enzyme leakage or degradation in the matrix.

A system similar to that described above has been devised for applying the enzymes glucose oxidase and urease onto adjacent ISFET gates using a photosensitive synthetic polymer consisting of poly(vinyl alcohol) (PVA) and styrylpyridinium or stilbazolium salt (See, Takatsu, I. and Moriizumi, T. in Sensors and Actuators 1987, 11, 309; Ichimura, K. United States Patent No. 4,272,620). Also, Moriizumi, T. and Miyahara, Y. in Sensors and Actuators 1985, 7, 1 and in an article published in Proceedings, Int'l. Conf. on Solid-State Sensors and Actuators, 1985, 148, describe the use of these photosensitive PVA membranes in methods which include spin-coating and injection into micro pools using a microsyringe. The poor long-term stability of the ISFET devices obtained with the spin-coated photopatterned PVA membranes was again acknowledged. The long-term sensitivity of the micro injected layers tended to be greater due partly to the greater thickness of these layers and the

correspondingly greater number of enzyme molecules remaining therein. However, in order to form the micro pools, into which the PVA mixtures are injected, a second photosensitive synthetic dry film must first be laminated onto the ISFET, irradiated, and developed to give the framed structures.

Other references exist which deal with the immobilization of urease onto electrochemical devices for performing a diagnostic test. A few of these methods involve pseudo-photolithographic procedures by which the enzyme is incorporated prior to or after the formation of the polymer layer (See, for example, Moriizumi, T. et al. in Sensors and Actuators 1986, 9, 373; Kimura, J. et al. in Proceedings, Int'l. Conf. on Solid-State Sensors and Actuators, 1985, 152; and Japanese Patent Nos. 56-115950 and 62-263457). These methods as described still fall far short of a viable microfabrication process.

Published Japanese Patent Application No. 62-235556 discloses a single sensor having three anodes and a common cathode. The sensor is made with the aid of azo-group-containing PVA, as the photo-bridged polymer. Glucose oxidase, galactose oxidase, L-amino acid oxidase and alcohol oxidase are among the enzymes claimed to be immoblized. No description is included which suggests the use of any material other than synthetic photosensitive polymers as the immobilization matrix. Furthermore, any teaching with respect to the manufacture of hundreds of identical reliable biosensors on a single wafer is not apparent.

2.2.2. SCREEN PRINTING METHODS

Screen printing of chemically sensitive materials as a step in a process for the mass production of chemical sensors has focused mainly on the deposited inorganic ceramic materials contained in certain organic binders. For example, Oyabu, T. et al., in J. Appl. Phys. 1982, 53(11), 7125, describe the preparation of thick film gas sensors using a

tin oxide paste and a screen printing method. The process includes a high temperature calcination step which is obviously not compatible with relatively fragile liquid membrane electrodes or enzyme-based sensors. Also, Cauhape, J.S. and co-workers, in Sensors and Actuators 1988, 15, 399, discuss the effect of mineral binders on the properties of screen-printed layers of semiconductor oxides. United States Patent No. 4,216,245, granted to Johnson, L.C., discloses a method for making printed reagent test devices using an offset or silk-screen dot printing method.

2.2.3. INK JET METHODS

Published Japanese Patent Application No. 62-223557 discloses a means for manufacturing an array of different enzyme layers on an integrted ISFET device. A hydrophilic porous film is established over the gate on the ISFET and then an ink jet nozzle is used to deposit enzyme onto the film. This process utilizes spray type technology with the fluid drop being first electrically charged and then fired from the nozzle. In this system the nozzle, fluid drop, and substrate surface are never in a contiguous physical contact. The diameter of the drops range from 20 to 100 micrometers. Also, published Japanese Patent Application No. 59-24244 discloses a similar membrane deposition process based on ink jet nozzle technology.

2.2.4. MICROSYRINGE METHODS

As already mentioned briefly, above, Moriizumi and Miyahara have employed microsyringe methods to inject synthetic polymer/enzyme mixtures into the gate regions of ISFET devices. These previously described techniques rely on ditches or pools to confine the dispensed fluid within the area of interest. In the article by Kimura and co-workers (Proceedings, Int'l Conf. on Solid-State Sensors and Actuators, 1985, 152, above), ISFET devices are described in

which various membrane compositions are deposited with the aid of a microsyringe. Again, a thick film resist polymer must be employed to delineate the area about the gate region of the ISFET device. In this manner, four types of membranes are applied and made separate from one another. No consideration is given to the volumetric profile of the drop (although a droplet value of 0.03 μL is given), its surface tension or the free energy of the surface of the device. Also, it is interesting to note that the injected enzyme (e.g., urease or glucose oxidase) solutions, which include a small amount of BSA, are immobilized within the gate region by the subsequent addition of a suitable amount of conventional glutaraldehyde solution.

United States Patent No. 4,549,951 granted to Knudson, M.B. et al. discusses the criticality of the shape and dimension of the ionophore layer but offers no insight for controlling these parameters. This reference teaches the use of a moat, carved around the perimeter of the electrode, to confine the membrane to that area. Some ion-sensitive membrane formulations are described.

Miyagi, H. et al., in articles which appeared in Technology Research Report of the Institute of Electronics and Communication Engineers of Japan 1986, 85(304), 31 and 1985 Pittsburgh Conference, 1058, describe two membrane deposition methods for ISFET devices: a screen printing method and a microsyringe method. The first employed a fine silica powder additive as a viscosity controlling agent and the second technique once again required a framed structure to hold in the membrane casting solution which was "poured into the frame with a microsyringe."

In a somewhat related method, Bousse, L.J. et al., in the Proceedings of the Second Int'l Meeting on Chemical Sensors 1986, 499, describe a lamination process in which a glass wafer is joined to a silicon wafer by anodic bonding. The bonding is carried out such that a chamber is formed

between the two wafers, the floor of which holds an electrochemical transducer. A laser is then used to drill holes through the ceiling of the chamber. Liquid membrane material is then introduced into the chamber, over the transducer, by applying a coat of the liquid membrane, placing the laminated wafer into a partially evacuated bell jar, and then venting the assembly to the atmosphere (thus forcing the liquid membrane material into the evacuated chamber).

It should be apparent that existing techniques for the uniform microfabrication of an array of chemical sensors are wholly inadequate and provide devices with specifications which are decidedly unsatisfactory. Furthermore, what methods exist have been developed mostly for application with ISFET devices. Unfortunately, ISFET and CHEMFET devices will always be plagued with disadvantages which are present intrinsically, such as their limitation to the detection of charged species only (See, for example, the review article by Flanagan, M.T. et al. in Anal. Chim. Acta 1988, 213, 23). The manufacture of miniaturized amperometric devices is even less established.

2.3. SILANE REAGENTS AND PERMSELECTIVE LAYERS

The use of silane coupling reagents, especially those of the formula $R'Si(OR)_3$ in which R' is typically an aliphatic group with a terminal amine and R is a lower alkyl group, to attach a macromolecule covalently to a solid support has been known for some time. For example, an article by Weetall, H.H. in Methods in Enzymology 1976, 44, 134-139, recommends heating the silane coupling agent to 115°C to promote the condensation of the agent with hydroxyl groups present on the surface of the solid support. A chemically modified platinum electrode has been described in which γ-aminopropyltriethoxysilane and glutaraldehyde were used in a step-wise process to attach and to co-crosslink

bovine serum albumin (BSA) and glucose oxidase (GOX) to the platinum surface (Yao, T. Analytica Chim. Acta 1983, 148, 27-33). These references do not contain any teaching that silane coupling reagents can be used for any other function besides promoting adhesion of overlaid materials or acting as a covalent anchoring agent.

Fujihara and co-workers, in J. Electroanalytical Chem. 1985, 195, 197-201, describes the use of a toluene solution of n-dodecyltriethoxysilane as a means for blocking the active sites of a catalytic gold electrode surface toward the reduction of hydrogen peroxide. The preparation of a permselective layer of variable thickness and its use to screen out undesired electroactive species while maintaining the high catalytic activity of the electrode surface are not disclosed or suggested.

Two published Japanese Patent Applications refer to the establishment of selective layers on non-microfabricated electrodes. Japanese Application No. 62-261952 describes the use of certain silane compounds, for the formation of a silicon layer which excludes the passage of uric and ascorbic acids but allows the permeation of hydrogen peroxide. Application No. JP 63-101743, pertains to a hydrogen peroxide permselective film which is derived from a high polymer film of poly(allylamine) crosslinked by the action of a suitable chemical agent. None of the references cited above discloses patterned permselective silane layers established on microfabricated devices.

2.4. FILM-FORMING LATICES

Particle latex materials and the distinct "film-forming" latices are old materials. Methods for producing film-forming latices by emulsion polymerization, their properties, and some of their uses have been reviewed (See, for example, Wagner and Fisher Kolloid Z. 1936, 77, 12; Vanderhoff, J.W. and Hwa, J.C. Polymer Symposia Wiley-

Interscience, New York (1969)). Additional references include: Whitley, G.S. and Katz, M.K. Indust. Eng. Chem. 1933, 25, 1204-1211 and 1338-1348; Matsumoto, T. Emulsions and Emulsion Technology Vol. II, Lissant, K.J. (Ed.), Marcel Dekker, New York (1974) Chapter 9; Encyclopedia of Polymer Science and Technology Vol. 5, John Wiley & Sons, New York (1966) pp. 802-859; Dillon, R.E. et al. J. Colloid Sci. 1951, 6, 108-117; and Sheetz, D.P. J. Appl. Polym. Sci. 1965, 9, 3759-3773.

A film-forming latex, ELVACE, containing a potassium chloride reference solution, has been applied over a reference microelectrode for an ISFET device (See, Sinsabaugh, S.L. et. al. Proceedings, Symposium on Electrochemical Sensors for Biomedical Applications, Vol. 86-14, Conan, K.N.L; (Ed.), The Electrochemical Society, Pennington, NJ (1986), pp. 66-73). This reference contains no teaching or suggestion that film-forming latices can be used as a medium containing anything other than an inorganic salt.

In summary, attempts by previous workers to manufacture viable biosensors with all the characteristics and specifications desirable in a reliable mass-produced microfabricated device have met with limited success. One of the more important aspects of wafer level processing, that of dimensional control both in the horizontal and vertical directions of a plurality of layered structures, which dimensional control in turn affects, inter alia, the uniformity of sensor performance, is irrevocably compromised when dicing and packaging occur prior to deposition of the biolayers. Manual handling is often necessitated by the fragility of the immobilizing or supporting layers and the labile nature of the bioactive molecules contained therein. Previous workers have had to resort to such methods, however. A flexible wafer level manufacturing process which utilizes superior materials and which makes possible the accommodation

of such sensitive bioactive molecules in a biosensor which can be tailored to a variety of clinical applications would be of major significance.

2.5. IMMUNOASSAY TECHNOLOGY

Immunoassays are sensitive diagnostic tools for the in vitro detection of a variety of antigens or antibodies and their association with diseases or other significant physiological conditions. In the early stages of developing immunoassay technology, a polyclonal antibody preparation bound to a solid phase was used in heterogeneous assays whereby a solution of labeled antigen was allowed to compete directly with antigen in a sample to determine the extent of bound labeled antigen or to detect the extent of antigen present in the liquid phase. This method provided a way for measuring the presence and quantity of antigen in the sample being analyzed.

Developments in immunoassay technology then led to non-competitive immunometric assays wherein a polyclonal antibody preparation bound to a solid phase was also employed. In these assays, a sample containing the target antigen was contacted with the solid phase to provide for antigen/antibody binding. Subsequent to an incubation period, the sample was removed from the solid phase and then the solid hase was washed to remove any unbound antigen. A solution containing labeled polyclonal antibodies (e.g. with a radionucleotide, enzyme, or fluorescent moiety) was then contacted with the solid phase. Unbound labeled antibody in the liquid phase was separated from the solid phase and bound labeled antibody (antibody:antigen:labeled antibody sandwich) on the solid phase was measured to determine the presence and/or concentration of antigen in the sample.

More rapid immunoassay procedures have also been developed. In these assays at least one of the two washing steps may be eliminated and incubation periods required to reach equilibrium may be shortened.

In the prior described processes the bound antibody is generally affixed to beads or small particles. The antibody can also be coated onto a surface. During the assay an incubation period is generally required of both the solid phase and labeled antibodies. A prolonged incubation period is particularly troublesome if results are needed quickly. Additionally, the long incubation periods and multiple washings have significantly limited the use of the assays to clinical laboratories, which have highly trained personnel and sophisticated equipment to undertake the assay. Consequently, there is presently a need for simpler and more rapid immunoassay protocols, and simpler apparatuses for use in emergency rooms, physicians' offices and even for in-home health care services.

2.6. COLORIMETRIC ASSAYS

Most existing assay protocols, including ELISA and enzymatic assays, provide for colorimetric detection. Generally these methods use a substrate which, itself, becomes a chromophore or which generates a chromophore, the chromophore is then detected spectrophotometrically. However, the spectrophotometric detection may have drawbacks because some measurements take an excessively long time or the sample mixtures are turbid. Some chromophores are also extremely unstable, thus, assay procedures involving non-chromogenic species may be useful.

Indoxyls and some of their derivatives have been employed as substrates in spectrophotometric assays. S. Cotson and S.J. Holt (Proc. Roy. Soc. B 1958, 148, 506) investigated their utilization in the production of tissue stains to identify alkaline phosphatase activity. P.L. Ely

and L.K. Ashman (Methods Enzymol. 1986, 121, 497) studied the use of bromo-chloro indoxyl phosphate as a substrate for determining the specificity of monoclonal antibodies to protein mixtures in alkaline-phosphatase-conjugated anti-immunoglobulin with immunoblots. J.J. Leary, D.J. Brigati and D.C. Ward (Proc. Natl. Acad. Sci. USA 1983, 80(13), 4045) utilized bromo-chloro-indoxyl phosphate for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized onto nitrocellulose i.e., bioblots. S.J. Holt and P.W. Sadler (Proc. Roy. Soc. B 1958, 148, 481) described the application of the conversion of indoxyl or a substituted indoxyl into the corresponding indigoid dye to cytochemical staining methods for the localization of cellular enzymes.

The kinetics of aerial oxidation of indoxyl and some of its halogen derivatives were studied by S. Cotson and S.J. Holt (Ibid. 1958, 148, 506) as part of their histochemical staining studies for work on enzymes. Their observations agree with the generally accepted view that such aerial oxidation reactions, involving free radicals, invariably result in the formation of organic peroxides or hydrogen peroxide, Waters, W.A., The Chemistry of Free Radicals, Oxford University Press, (1946). The aerial oxidation of indoxyls was studied utilizing spectrophotometric methods. All of the above references, exploited the chromogenic properties of indigoid compounds derived from indoxyls.

Examples of other chromogenic applications of the oxidative conversion of indoxyl compounds to indigoid dye have included: an indigogenic reaction for alkaline and acid phosphatase histochemical demonstration in disk electrophoresis (E. Epstein, P.L. Wolf, J.P. Horwitz, and B. Zak in Am. J. Clin. Pathol. 1967, 48(5), 530); the comparison of simultaneous azo-dye coupling methods and an indigogenic reaction for alkaline phosphatase in polyacrylamide disc gels (T.F. Savage, E.C. Smith, and Collins in Stain Technol. 1972, 47(2), 77); protein blotting principles and applications

(J.M. Gershoni and G.E. Palade in Anal. Biochem. 1983, 131(1), 1); a sensitive method for staining proteins transferred to nitrocellulose sheets (Z. Wojtkowiak, R.C. Briggs, L.S. Hnilica in Ibid. 1983, 129(2), 486); visualization of antigenic proteins on Western blots (D.A. Knecht, R.L. Dimond in Anal. Biochem. 1984, 136(1), 180); a rapid sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots (M.S. Blake, K.H. Johnston, G.J. Russel-Jones, and E.C. Gotschlich in Ibid. 1984, 136(1), 175); immunoconcentration - a new format for solid phase immunoassays (G.E. Valkirs and R. Barton in Clin. Chem. 1985, 31(9), 1427); the use of alkaline phosphatase conjugated anti-immunoglobulin with immunoblots for determining the specificity of monoclonal antibodies to protein mixtures (P.L. Ey and Leonie K. Ashman in Methods Enzymol. 1986, 121, 497); and work involving the coupling of redox and enzymatic reactions which has been found to improve the sensitivity of the ELISA-spot assay (C. Franci and J. Vidal (J. Immunol. Methods 1988, 107(2), 239).

Again, all of the preceding references rely exclusively on the spectral properties of bromo-chloro-indoxyl phosphate as a colorimetric substrate.

2.7. ELECTROCHEMICAL SENSORS AND ASSAY

There has recently been a significant interest in the construction of electrochemical sensors, so-called immunosensors, that are capable of integration into immunoassay protocols. M.J. Green (Philos. Trans. R. Soc. Lond. B. Biol. Sci. 1987, 316(1176), 135) has reviewed several immunoassays that incorporate electroactive labels for the amperometric or potentiometric detection of assay products. However, the translation of working laboratory prototypes, as reported in the book, Biosensors: Fundamentals and Application, edited by A.P.F. Turner, I.

Karube, and G.S. Wilson, Oxford University Press, 1987, into common commercially available articles, has been impeded by the absence of appropriate manufacturing protocols.

A specific example of electrochemical detection as an alternative to color detection is described in Anal. Chem. 1984 56, 2355. The reference discloses an assay in which an enzyme label converts an electroinactive compound to a detectable electroactive compound. The electroactive compound, phenol, is oxidized at a potential of +750 mV. However, the method is not generally applicable since other electroactive components are present in blood or serum which are also oxidizable at this potential.

A very recent reference which illustrates the prevailing notions ingrained in those skilled in the art of "immunoelectrochemical sensing" is that by Rosen, I. and Rishpon, J. in J. Electroanal. Chem. 1989, 258, 27. In this article, an enzyme is used as a label which is capable of transforming a substrate, which is not electroactive, to one which is. In particular, alakaline phosphatase is the enzyme employed. Several substrates are examined, including phenylphosphate, p-nitrophenylphosphate, and p-aminophenylphosphate. In the electrochemical detection method described, the alcohol products, resulting from the hydrolysis reaction catalyzed by the enzyme (i.e., phenol, p-nitrophenol, and p-aminophenol, respectively), themselves, are detected. The viability of detecting other electroactive species, besides the transformed substrate, is not suggested and, indeed, is never contemplated.

Also, European Patent Applications Nos. 0247796 and 0270206 describe methods for conducting immunoassays which involve primarily moveable magnetic particles to which are bound immunoactive molecules. Enzyme conjugates are described which generate electroactive species such as H_2O_2. However, the principal means of detection involves

chemiluminescence and, in any event, indoxyl compounds are not mentioned and no microfabricated sensing devices useful in performing immunoassays are disclosed.

3. SUMMARY OF THE INVENTION

The present invention relates to wholly microfabricated biosensors and various processes for the mass microfabrication thereof. The microfabrication processes establish a plurality of thin films and related structures over a planar wafer in a fashion which allows exemplary reproducibility and control over the dimensional features of the overlaid structures. In the present invention, such reproducibility and dimensional control have been realized at the wafer level for the mass production of chemical sensors, which sensors incorporate biologically active macromolecules and other reagents necessary for the conversion of selected analyte molecules to more readily detectable species.

This invention also relates to novel electrochemical assay procedures and to novel wholly microfabricated biosensors useful in determining the presence and/or concentration of biological species (analytes) of interest. The invention also relates to the novel use of a substrate (hereinafter the "substrate") that does not undergo detectable electrochemical oxidation or reduction but which undergoes a reaction with a substrate converter producing changes in the concentration of electroactive species. These changes are measured and related proportionately to the concentration of the analyte of interest. Additionally, the invention pertains to methods for making the biosensor.

The assay procedures and biosensor of this invention are particularly exemplified as being useful in effecting immunoassays. Such immunoassays are also exemplified wherein the substrate convertor is an enzyme that hydrolyzes the substrate. This hydrolyzed substrate can

then undergo reactions which produce changes in the concentration of electroactive species (dioxygen and hydrogen peroxide) which are electrochemically detected with the biosensor, a ligand/ligand receptor-based (LLR-based) biosensor in this instance. Both sandwich and competitive assays can be effected using the procedures and LLR-based biosensors of this invention. In these assays, one embodiment of the present biosensor comprises a catalytic electrode and optional reference electrode (base sensor), an adhesion promoter layer overlaid on the biosensor, and a bioactive layer that is immobilized on the adhesion promoter layer, which bioactive layer is a receptor (first member) of the immunological analyte of interest.

The wholly microfabricated biosensor of the present invention comprises a substantially planar wafer on which a first structure comprising a suitable base sensor is established. Additional structures are then established over the resulting base sensor, which additional structures include a semipermeable solid film or permselective layer capable of acting as a barrier against interfering chemical species while allowing the transport of smaller detectable chemical moieties of interest. These detectable chemical moieties are typically electroactive molecules and may include low molecular weight ionic species. The semipermeable solid film may further comprise compounds or molecules which may serve to sensitize the base sensor to a preselected ionic species (e.g., ammonium ion). Furthermore, such permselective layers may also function as adhesion promoters by which the preselected ligand receptor may be immobilized to the wholly microfabricated LLR-based biosensor embodiment of the present invention.

Most noteworthy are the support matrices described in the instant invention which matrices possess the physical and chemical features necessary to support the various bioactive

molecules that constitute the principal means for converting the particular analytes in a given analytical sample into detectable and/or quantitatively measureable species. Techniques are disclosed for localizing or patterning said matrices on certain desired areas of the wholly microfabricated biosensor which allow for the optimum control over dimensional features of the biolayers as well as the versatility to accommodate a wide range of bioactive molecules.

Additionally, the present invention also discloses materials which serve, in particular embodiments of the instant biosensor, as overlaid structures for the attenuation of the transport of selected analyte species which are present in high concentrations in the sample. Such analyte attenuation (AA) layers allow for a linear sensor response over a wider range of analyte concentrations than would be observed in the absence of an AA layer. Furthermore, the overlaid AA layer, which is preferably derived from a siloxane/nonsiloxane copolymer, is capable of excluding very large molecules or other contaminating constituents of the sample whose direct contact with the underlying structures would result in interference with or fouling and an eventual reduction in the reliability of the biosensor.

If the AA layer is of the appropriate structure and composition, it may also function as a gas permeable membrane. In certain embodiments of the present invention, such a gas permeable membrane has the practical advantage of allowing only very small molecules to pass through. The gas permeable membrane also insulates the immediate environment of the electrode portion of the biosensor from external fluid turbulence. Thus, the measurements performed by the preferred LLR-based sensor is rendered free of flow dependence.

The AA layer of the instant invention is established on the substrate wafer or any intervening structures with the kind of dimensional, localized, and geometric control which is compatible with the other steps in the overall microfabrication process of the instant invention and the notion of an automated, wafer-level mass-production of biosensors.

Quite apart from the AA layer mentioned above, a semipermeable solid film which is able to function as a molecular weight-sensitive transmissive film is among the layers which can be established by the methods of the present invention. Depending upon the composition and final thickness of this semipermeable solid film, also referred to as a permselective layer, molecules having molecular weights above a given threshold can be effectively excluded from entering and diffusing through such a film. As a general illustration of the function and utility of this permselective layer, molecules having a molecular weight of about 120 or above are effectively blocked by a solid film having a thickness of about 5 to about 10 nm. Varying degrees of control over the size of the molecules excluded and the rates of transport of smaller molecules which are able to diffuse through the solid film can be obtained with solid films having a thickness in the range of about 2 to about 50 nm. With certain types of materials, these permselective layers may be as thin as 1 nm or may be as thick as 100 nm.

This film may be established on the substrate wafer or any planar analyte-sensing device in a number of ways but most conveniently as an initial liquid film, comprising a silane compound mixed with a suitable solvent, which is spin-coated across the wafer. The silane compound has a formula, $R'_nSi(OR)_{4-n}$, in which n is an integer which may be 0, 1, or 2, R' is a hydrocarbon radical comprising 3-12 carbon atoms, and R is a hydrogen radical or a lower alkyl

radical comprising 1-4 carbon atoms. Preferably, the solvent contains an amount of moisture sufficient to hydrolyze the alkoxy groups of the silane compound, if present. The wafer bearing the liquid film is then heated to a temperature of about 90-250°C for a period of time effective to form the solid film. Typically about 5 to 30 minutes of heating at this temperature is required. The non-volatile content of the initial silane solution determines the final thickness of the permselective layer which can thus be controlled.

If desired, this permselective layer may be formed at specific preselected areas of the device by means of photolithographic processing techniques. Techniques such as "lift-off" and use of a photoresist cap in combination with a plasma-etching or, alternatively, a wet-etching step may thus be employed to define the location and configuration of the semipermeable solid film. The initial liquid silane mixture, much like the majority of other liquid mixtures disclosed for use in the present invention, can also be microdispensed at multiple preselected areas of the sensing device. Such microdispensing of fluid media may be performed automatically and in uniform predetermined quantities by a computer-controlled syringe interfaced with the controlled movements of a vacuum chuck holding the substrate wafer. Such microdispensing techniques are consistent with a microfabrication method and is discussed in further detail below.

Thus, in an amperometric electrochemical sensing device, interfering electroactive species having a molecular weight above a desired threshold (e.g., above 120) may effectively be excluded from interacting with the catalytic electrode surface by employing the permselective silane layer of the present invention. Such a permselective layer, however, allows lower molecular weight electroactive species, like dioxygen and hydrogen peroxide, to undergo a redox reaction with the underlying electrode surface.

In a potentiometric biosensor, a polymeric material having functional groups and chemical properties conducive to the further incorporation of certain ionophoric compounds may be used as a semipermeable ion-sensitive film which is established on the indicator electrode of said sensing device. The development of a potential at the electrode-film interface depends on the charge density, established at equilibrium, of some preselected ionic species. The identity of such ionic species is determined by the choice of the ionophore incorporated in the semipermeable film. An enzyme which is, in turn, immobilized in the novel biolayers described herein catalyzes the conversion of a particular analyte species, present in the analytical sample, to the preselected ionic species.

The permselective layers discussed above are selected for their specificity to the ionic electroactive chemical species which are produced by chemical processes taking place in the overlaid structures referred to herein as the biolayer. The chemical process which converts a selected analyte species or exogenous reagent into an ionic electroactive chemical species is effected by at least one biologically active molecule, such as an enzyme, which is incorporated in the biolayer. The support matrices of the biolayer and methods of the instant invention help to stabilize the bioactive molecules against degradation caused by further processing, storage, handling, or exposure to analyte or reagent compositions. These support matrices must retain a certain degree of porosity such that analytes of interest may freely diffuse through the matrix and undergo chemical transformation. Because the wholly microfabricated biosensors of the instant invention are likely to be stored essentially dry, such porosity will also help in the initial wet up and calibration sequence used to prepare the biosensor for the actual analytical procedure. If the sensitivity of the bioactive molecule so dictates, the support matrix is

also able to accept and immobilize enzymes introduced, for instance, from a solution after the matrix has been established locally and/or photolithographically patterned and developed. In any event, a sufficient amount of biocatalyst and/or ligand receptor must be present in the biolayer to overcome any inactivation due to subsequent processing or handling, or due simply to the passage of time during storage. Sufficient biocatalyst/ligand receptor should also be immobilized to provide a favorable condition for the efficient and ready conversion of infusing analyte molecules. Thus, the biolayer of the present invention comprises a sufficient amount of a bioactive molecule capable of selectively interacting with an analyte species and a support matrix in which the bioactive molecule is incorporated, which matrix may be a photoformable proteinaceous mixture, a film-forming latex, or combinations of these materials. As mentioned previously, the analyte species must be able to freely permeate through the support matrix and interact with the bioactive molecule contained therein. A variety of additives disclosed above may be added to the support matrices to further achieve desirable functional and structural characteristics not inconsistent with the objectives of the present invention.

As alluded to earlier, these biolayers are established with the dimensional and geometric control characteristic of wafer level manufacturing procedures. Thin-film techniques, spin-coating, use of photoresist materials, masking, exposure to radiant energy, and developing methods can be utilized for the majority of biologically active molecules. For the preceding techniques, photoformable proteinaceous mixtures are most conveniently used as the support matrix. If necessary, however, extremely labile enzymes may be introduced later, after the photodefined structures have been established. Such support matrices may also serve as electrolyte layers, as well as the photoresist layers over

which the ligand receptors of interest may be immobilized. Preferably, the immunoreactive species or ligand receptors are introduced after the photodefined structures have been established.

Alternatively, film-forming compositions, which may include synthetic as well as naturally-derived polymeric materials, can be used to establish the solid matrices especially when microdispensing is the method of choice for establishing the biolayers. Combinations of photoformable gelatins and film-forming latices may be employed. Again, reagents or additives may be incorporated into these layered structures as might be dictated by the particular application or analysis at hand.

The present invention thus relates also to a method of establishing a dispensed layer onto a substantially planar surface. This method succeeds in providing layers having predictable and reproducible dimensions by adjusting the composition of a fluid to be dispensed, until its surface tension and viscosity characteristics are optimized, providing a movable microsyringe assembly, and using the assembly in a manner which allows for close control over the amount of fluid dispensed. Furthermore, the microdispensing method disclosed herein may be coupled effectively with known techniques for altering the free energy of a given surface such that the physical characteristics of the established layer (e.g., contact angle, thickness, volume, or area) may be tailored even further to accommodate a desired application.

Additional layers may also be desirable as mentioned previously to enhance the sensitivity and response time of the device, extend the range of a linear response, and increase the durability of the overlaid structures. In the case of an analyte attenuation layer, certain copolymers comprising siloxane and nonsiloxane units may be employed advantageously. These materials may also be layered or

established at a given thickness, anywhere from about 5 to about 500 nm, and may be localized by photolithographic methods. Typically, the analyte attenuation layer should have a thickness sufficient to attenuate the transport therethrough of analyte species having a molecular weight of about 120 or more. As stated previously, these AA layers may also be established at a thickness sufficient to provide a gas permeable membrane. In connection with the photoforming step, a "resist cap" method may be employed, for instance, using a type of photoformable proteinaceous mixture which is also a nonbarrier (i.e., it does not impede or exclude the transport of relevant analyte species).

These and additional objects of the instant invention are apparent from the disclosures and examples included herein.

4. BRIEF DESCRIPTION OF THE FIGURES

The invention may be better understood if reference is made to the accompanying drawings. These drawings, especially the schematics of the wholly microfabricated sensor structures, are qualitative and topological in nature and are not meant to convey absolute dimensional relationships between the various layers or parts of the biosensor.

FIG. 1 Top elevation of a differential amperometric glucose sensor on a 6 x 3 mm rectangular silicon chip. The significance of the different layers is discussed further in Section 5.1, below. The same general configuration may also be employed for the LLR-based biosensor embodiment of the present invention. Alternatively, FIG. 1 may also illustrate a differential amperometric LLR-based biosensor on a 6 x 3 mm rectangular chip. The various areas/layers of the chip refer to contact pads (1), signal line (2), passivation (3), silver/silver chloride reference electrode (4), metal

catalytic indicator electrode (5), adhesion promoter (6), or localized adhesion promoter (7), coupling means (8), and ligand receptor layer (9).

FIG. 2 Side elevation of one, of the glucose sensor pair of FIG. 1 with surrounding silver/silver chloride reference electrode.

FIG. 3 Side elevation of a potentiometric blood urea nitrogen (BUN) sensor and reference electrode.

FIG. 4 Top elevation of the sensor of FIG. 3 showing an array of different biosensors on a single chip.

FIG. 5 Current output (in namps) of the present glucose sensor (oxidation/reduction of hydrogen peroxide) as a function of electrode potential (mV) using a 20 mM glucose in HEPES buffer sample (O) or HEPES buffer only (X).

FIG. 6 Current output (in namps) of the present glucose sensor as a function of glucose concentration (mM) in the sample.

FIG. 7A An alternative embodiment of an amperometric oxygen sensor of the instant invention which utilizes a gas permeable layer. This configuration is also well-suited for the LLR-based biosensor application of the present invention. In the LLR-based embodiment, the electrolyte layer (12) is also the first photoresist layer; the gas permeable membrane (8') (also referred to as the AA or gas permeable layer) is established over the first photoresist layer; and the photoresist cap (9, also the second photoresist layer) is present above the AA layer.

FIG. 7B The diagram illustrates a configuration in which the gas permeable layer substantially encloses an underlying electrolyte layer (or first photoresist layer in the LLR-based biosensor embodiment).

FIG. 8A An alternative configuration of a glucose biosensor based upon the dioxygen sensor described herein.

FIG. 8B A Ligand/Ligand Receptor-based (LLR-based) biosensor with immobilized ligand receptor or immunoreactive species (45). The underlying sensor configuration is derived from that of FIG. 7B. This illustration also employs coupling means (40) to immobilize the active species (45).

FIG. 9 Uniformity of the response of three blood urea nitrogen (BUN) sensors, wholly microfabricated by the process of the present invention, to a change in the ammonium ion concentration of an aqueous sample from 2 to 20 mM.

FIG. 10 Response of the present BUN sensor to a change in the urea concentration of an aqueous solution from 1 to 10 mM.

FIG. 11 Response of the present BUN sensor to a whole blood sample spiked with urea.

FIG. 12 An illustration of one possible configuration of the automated microsdispensing system of the present invention in which syringe (5), holding the material to be dispensed, is attached to a means, (8), for controlling its displacement in the direction, z, while wafer, (2), is held to a vacuum chuck, (1), whose movement in all directions is, likewise, controlled by an automated, computerized means. The system may also include a visual means for alignment (e.g., video camera equipped with a raticle alignable with appropriate alignment features on the wafer).

FIG. 13 An alternative configuration of the automated microdispensing system comprising multiple syringe holders, (7). The syringes are inserted into openings, (13), and the vacuum chuck and wafer are positioned below the ring, (11), and through large opening, (12).

FIG. 14 A schematic rendering of a typical sandwich assay performed using the present invention is shown. An immobilized ligand receptor (the first member) is positioned near the surface of the biosensor and encounters an analyte molecule (the ligand). The ligand binds to the receptor and is subsequently attached by an antibody-enzyme conjugate (the

labeled antibody or the second member). A substrate is added next which undergoes a chemical transformation mediated by the enzyme (the label or substrate converter). The resulting intermediate product then undergoes a cascade of reactions involving the consumption of dioxygen and the production of hydrogen peroxide (both O_2 and H_2O_2 are detectable, electroactive species) and final product (indigo when the initial substrate is an indoxyl derivative).

FIGS. 15a-15e illustrate the effect of pretreating the electrode surface to alter its surface free energy characteristics. The contact angle, θ, of a microdispensed fluid, and eventually, the thickness of the membrane layer above the electrode, is thus controlled.

FIGS. 16a-16c show various embodiments of microdispensed biolayers including one which has a large contact angle (FIG. 16a), one with a small contact angle (FIG. 16b), and one which has subsequently been subjected to a photopatterning step (FIG. 16c).

5. DETAILED DESCRIPTION OF THE INVENTION

The present manufacturing method is directed to the mass production of biosensors having predictable, uniform response characteristics and which biosensors are useful in a clinical setting for the convenient and real-time detection and quantitative measurement of selected analyte species. The integrated biochemical sensing device is formed on a transducer array by establishing discrete layered structures which are robust and possess a controlled degree of porosity, at least one of which layered structures is capable of immobilizing one or more biologically active species. The term biologically active or bioactive molecule is used to encompass ionophores, ion-exchangers, enzymes, antibodies, antigens, lectins, neurochemical receptors, oligonucleotides, polynucleotides, polypeptides, molecules of DNA, molecules of RNA, proteins, glycoproteins, metalloproteins, cofactors,

immunoglobulins, and other macromolecules of physiological significance including mixtures or active fragments or subunits thereof. The term biocatalyst may also be employed especially with reference to an enzyme, enzyme-complex or mixture thereof. In general, a broad class of ligand receptors may be immobilized and used in the present biosensors.

The steps comprising the method of this invention, as well as the materials disclosed which may be used to establish the discrete layered structures of the present microfabricated device, retain a surprising degree of flexibility and versatility such that a wide range of analyte species may be selectively examined. Furthermore, the microfabricated sensing device, or biosensor for short, is available for the analysis of most liquid samples including biological fluids such as whole-blood, lymph, plasma, serum, saliva, urine, stool, perspiration, mucus, tears, cerebro-spinal fluid, nasal secretion, cervical or vaginal secretion, semen, pleural fluid, amniotic fluid, peritoneal fluid, middle ear fluid, joint fluid, gastric aspirate or the like. It should also be understood that solid or dessicated samples may be dissolved in an appropriate solvent to provide a liquid mixture suitable for analysis.

A second and related part of the invention is a means of photodefining optional additional layers over the active layer to protect it from contact with any deleterious components of the analytical sample or solution containing the analyte (i.e., the species to be analyzed or determined). In certain cases, such additional layers serve to attenuate the transport of the selected analyte into the biologically active layer, particularly when the selected analyte is present in high concentration in the sample. In so doing, the effective range of analyte concentrations in which the biosensor has a linear response is extended to higher values. Such analyte attenuation (AA) layers can also impair the

responsiveness of the resulting sensing device and, therefore, their thickness must be carefully considered and controlled. Where the concentrations of selected analytes in the sample are not so great as to result in a nonlinear sensor response, such AA layers need not be established.

In certain embodiments of the invention, this AA or gas permeable layer, in addition to attenuating the transport of certain analytes or electroactive species, is also responsible for "insulating" the response of the sensor against the effects of sample turbulence or flow. Having a sensor response which is less sensitive to the external sample flow provides a more reproducible, reliable signal, and such a configuration is preferred particularly for the LLR-based biosensor embodiments described further herein.

Furthermore, a semipermeable solid film has also been discovered, which solid film may be established and patterned (photodefined) over preselected areas of a chemical sensing device. This permselective layer is able to act as a barrier against the intrusion of interfering electroactive species while the desired electroactive species may freely diffuse through said film. In a particular embodiment of this invention, the permselective layer is derived from a silanizing agent. Typically, a relatively stable silane precursor is dissolved or mixed in a solution which is able to hydrolyze at least two of the groups attached to the central silicon atom of the silane precursor. The resulting reagent solution is then established as a film across the wafer or localized over preselected areas of the wafer or base sensor. A semipermeable or permselective layer is then obtained under carefully controlled heating conditions. The permselective properties of the layer are governed, in part, by the thickness of the layer which is, in turn, dependent upon the nature and amount of silanizing agent employed as well as the method used to establish the film. Whenever desirable, mixtures of silanizing agents may be employed.

By eliminating most interfering electroactive species, fewer corrective measures are needed and the result is an operationally simpler device. Moreover, the base transducer, which frequently comprises a catalytic metal surface, may be heated in the presence of said permselective layer, to temperatures in excess of about 150°C to about 250°C. This deliberate heating step provides an enhanced responsiveness of the base sensor to the primary electroactive species of interest (e.g., hydrogen peroxide or dioxygen) while maintaining the exclusionary nature of the solid film towards interfering electroactive species of higher molecular weight (e.g., uric acid or ascorbic acid).

In particular embodiments of the present invention, the transduction of the analyte concentration into a processable signal is by electrochemical means. These transducers may include amperometric, potentiometric, or conductimetric base sensors. However, the microfabrication techniques and materials of the instant invention may clearly be applied to other types of transducers (e.g., acoustic wave sensing devices, thermistors, gas-sensing electrodes, field-effect transistors, optical and evanescent field wave guides, and the like) fabricated in a substantially planar manner. A useful discussion and tabulation of transducers which may be exploited in a biosensor as well as the kinds of analytical applications in which each type of transducer or biosensor, in general, may be utilized is found in an article by Christopher R. Lowe in Trends in Biotech. 1984, 2(3), 59-65. The disclosures and descriptions contained in this Lowe article are incorporated by reference herein. Of the three electroanalytical techniques mentioned earlier, the potentiometric and amperometric techniques are preferred because the output signal may most easily be related directly to the response of the base sensor to a particular analyte.

Specific examples directed to the production of potentiometric and amperometric type biosensors are found in the Examples Section of the present disclosure.

Herein are illustrated the various aspects of the invention which combine to yield a viable manufacturing process for an array of biosensors, for use in the analysis of undiluted biological samples. Preferred embodiments of an amperometric glucose sensor and a potentiometric urea sensor are described further. These sensors are useful for analyzing the concentration of glucose and urea, respectively, present in a given sample (e.g., venous blood). Various other sensors are likewise disclosed, including an embodiment especially adapted to perform immunology or affinity-based analyses, for the detection and measurement of analyte molecules of physiological significance, along with descriptions of modified configurations made possible by the present discoveries.

More particularly, this invention also relates to novel electrochemical assay procedures and to a novel wholly microfabricated LLR-based biosensor useful in determining the presence and/or concentration of selected biological (analytes) species of interest. This aspect of the invention relates to the discovery that a non-electroactive substrate (hereinafter the "substrate"), which does not undergo detectable electrochemical oxidation or reduction at an electrode at operating potentials which are accessible in aqueous based systems, but which undergoes a reaction with a substrate converter to form an unstable intermediate. The intermediate undergoes rapid auto-oxidation causing changes in the concentration of electrochemically detectable species. These detectable species include dioxygen and hydrogen peroxide. The changes are measured and related to the concentration of the analyte of interest.

Such novel assay procedures and LLR-based biosensor are useful in detecting the presence of, or monitoring the level of, one or more analytes in a mixture at a particular concentration, in the presence of potentially interfering substances. As mentioned previously the presence or absence of a particular analyte is determined from the extent of a specific binding interaction between an analyte and the first member (a capture receptor). The binding interaction, itself, is detected when a second member (the detection receptor), which is conjugated with a label (substrate converter), reacts with a substrate to give rise to the production and/or consumption (change in concentration) of detectable species (e.g., hydrogen peroxide or dioxygen); See Figure 14. These concentration changes are electrochemically detected using the apparatus and assaying procedures of this invention. In particular embodiments of the present invention, labeled analyte species may also be employed in "competitive assay" procedures.

In a preferred embodiment of the present invention, a conjugated enzyme is used as the substrate converter (label) to effect a change in the concentration of the electroactive species. The enzyme may be conjugated to the analyte. Any change is detected electrochemically and related to the analyte of interest. In particular, the invention pertains to the preferred use of the enzyme alkaline phosphatase as the label and an indoxyl phosphate derivative as the substrate. However, it is apparent to one of ordinary skill in the art that the present invention is not so limited. In the general case, an esterase or hydrolase can be used to hydrolyze any indoxyl ester as long as the product undergoes rapid auto-oxidation. In yet other cases, the reaction of the enzyme with the substrate itself produces such a change in the concentration of electroactive species.

More particularly, but not exclusively, the invention is concerned with electrochemical immunoassay procedures and devices to determine analytes of interest. In this regard, an enzyme-labeled antibody or an enzyme-labeled antigen reacts with a substrate to effect a change in the concentration of electroactive species that is susceptible to electrochemical detection. In addition, since the enzyme-labeled antibody or enzyme-labeled antigen species is bound respectively to a complementary antigen or antibody species in a biological sample, the electrochemically detected enzyme reaction, therefore, provides for the qualitative or quantitative determination of species of interest. Specifically, this invention pertains to the reaction of the non-electroactive indoxyl phosphoric acid ester, which is the substrate, with an alkaline-phosphatase labeled goat anti-human Immunoglobulin G (antibody) or with an alkaline-phosphatase labeled theophylline (antigen). These two reactions are associated with sandwich-type or competitive-type immunoassay methods, respectively.

It should be noted that the invention exemplified herein also extends to other assay systems. Theoretically, any ligand/ligand receptor pair in which at least one member can be immobilized onto the present biosensor can be incorporated into an assay procedure. Table II (Section 5.2.2) lists just a few examples of such ligand receptor/ligand pairs. Furthermore, other substrates, whose reaction with the label, or subsequent auto-oxidation, produces and/or consumes dioxygen or hydrogen peroxide may be readily contemplated. The invention is, therefore, not limited to the use of a phosphatase enzyme as the label, as already mentioned, because other hydrolases and labeling enzymes which are capable of reacting with a reagent to effectuate a change in the concentration of electroactive species are equally suitable (See, for example, Table II). Again, it must be stressed that, while the invention is

described with reference to immunoassay procedures and with an immunological assay apparatus, it is also submitted that other types of specific binding reactions, such as those between other complementary binding species (e.g., enzyme/metabolite, lectin/polysaccharide, and nucleic acid oligomer/anti-oligomer) are also detectable employing the aforesaid electrochemical assay procedures and devices (See, for example, Tables II and III).

The present invention, therefore, provides processes and devices for performing simply and rapidly analyte-receptor assays, for example immuno- and immunometric assays, which utilize an electrochemical sensor, and which do not require lengthy incubation steps. The electrochemical procedure and apparatuses described herein for the detection of phosphatase (label) activity are also highly specific and relatively sensitive. In addition, chromogenic and turbidimetric interferences are eliminated due to the nature of the detection system. The use of enzyme labels in the assay together with a non-electroactive enzyme substrate also potentially facilitates the extension of known specific binding assays to greater levels of resolution than those previously accomplished, usually without the requirement of pretreatment of samples to remove interfering substances. More particularly, the assay of an analyte in the nanomolar and above concentration range is thereby achieved.

5.1. AMPEROMETRIC GLUCOSE SENSOR

The wholly microfabricated glucose sensor of the present invention comprises a silicon substrate on which is established thin-film structures which make up an amperometric electrochemical transducer, or base sensor. In a particular embodiment of the present invention, succeeding overlaid structures can be described as (i) a semipermeable solid film or permselective layer, superimposed over at least a portion of the base sensor, whose function is to promote

the adhesion of succeeding layers over the base sensor and most importantly to prevent interfering electroactive species which are usually present in venous blood or other biological fluid samples from reaching the catalytic electroactive surface of the base sensor; (ii) a biolayer, superimposed over at least a portion of the permselective layer, in which is incorporated a sufficient amount of the bioactive molecule, in this case the enzyme glucose oxidase; and (iii) a layer responsible for attenuating the transport of the analyte species, in this case glucose, from the sample to the biolayer. The analyte attenuation (AA) layer thus limits the amount of glucose which reaches the enzyme to a given fraction of the bulk concentration of glucose in the sample.

The term "incorporated" as used herein is meant to describe any state or condition by which the material incorporated is held on the outer surface of or within a solid phase or supporting matrix. Thus, the material "incorporated" may, for example, be immobilized, physically entrapped, attached covalently to functional groups of the matrix, or adsorbed onto the porous surface thereof. Furthermore, any process, reagents, additives, or molecular linker agents which promote the "incorporation" of said material may be employed if these additional steps or agents are not detrimental to, but are consistent with the objectives of the present invention. This definition applies, of course, to any of the embodiments of the present invention in which a bioactive molecule is "incorporated."

The succeeding overlaid structures are preferably confined to the locality of the electroactive surface of the indicator electrode of the base sensor. These structures may be localized by means of micro-dispensing or photolithographic techniques. An additional layer which comprises a photoresist cap may optionally be present over the AA layer as a consequence of the photoforming steps.

This outermost cap can be established such that it does not act as a barrier to any species of interest, if any, and, therefore, need not be removed.

The basic chemical and electrochemical transformations on which the analytical value of the present device is premised include the convertion of glucose to gluconolactone by the action of the enzyme glucose oxidase (GOX):

$$\text{glucose} + O_2 \xrightarrow{GOX} \text{gluconolactone} + H_2O_2 \qquad (1)$$

As indicated by Eq. 1, this transformation is accompanied by the concurrent reduction of dioxygen to hydrogen peroxide. Both dioxygen and hydrogen peroxide are electroactive species which can undergo redox reactions on the electrocatalytic surface of the indicator electrode of the base transducer. Other electroactive species (such as Na^+, K^+, Ca^{2+}, NH_4^+, etc.) do not undergo a redox reaction per se but do promote a change in the potential at the electrode interface (See, e.g., the potentiometric device in Section 5.3). Thus, by applying the appropriate potential across the indicator electrode surface, with respect to a reference electrode, one of the following electrochemical reactions

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O \qquad (2)$$

$$H_2O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O \qquad (3)$$

$$H_2O_2 \longrightarrow O_2 + 2e^- + 2H^+ \qquad (4)$$

can take place, all of which result in the consumption of the electroactive species and the production of a measurable positive or negative current. Of the three reactions listed above, Eq. 4 is preferred in the present embodiment because it releases an equivalent of dioxygen per equivalent of hydrogen peroxide amperometrically measured. The dioxygen produced helps to maintain an adequate supply of dioxygen available for the enzymatic process of Eq. 1. The potential

required for the oxidation of hydrogen peroxide is about +300 to about +600 mV, preferably +350 mV, vs. silver/silver chloride reference electrode. The current produced as a function of the indicator electrode potential of a glucose sensor of the present invention is illustrated in Fig. 5 for test samples comprising a HEPES buffer (X) and a 20 mM glucose solution in HEPES (Sigma Chemical Company) buffer (O). An increase in the current is observed as the indicator electrode potential is increased from 150 to about +350 mV for this particular glucose sensor. Further increases in the indicator electrode potential result in a nearly level response showing that, in the steady-state, the magnitude of the current produced is limited eventually by the amount of glucose analyte which is transported through the AA layer. This steady-state range extends from about +350 to about +600 mV as shown in Fig. 5. Conversely, a negative current is observed with a more negative indicator electrode potential as electrons are used up in the reduction of hydrogen peroxide to water (Eq. 3). Once again a limiting steady-state negative value for the current is reached at a certain negative potential (about -100 mV) and remains relatively constant through further increase in the negative value of the indicator electrode potential. One preferably operates the indicator electrode at the plateau to avoid large incremental changes in the current produced by small changes in the indicator electrode potential versus the reference electrode.

The current generated as a result of the preceding electrochemical reactions may be related ultimately to the concentration of glucose present in the sample. In an amperometric sensor, such as the glucose sensor, the measured variable (i.e., the current, i) is related to the flux of the electroactive species at the electrode surface (at a distance, x = 0) by Faraday's laws, in combination with Fick's Law of diffusion (Eq. 5):

$$i = nFAD_p \left.\frac{\partial [P]}{\partial x}\right|_{x = 0} \quad (5)$$

where, n is the number of electrons involved in the fundamental electrochemical reaction at the electrode, F is Faraday's constant, A is the area of the electrode, and Dp is the diffusion coefficient of the electroactive species, P. In the steady-state, the rate of the enzymatic reaction in the biolayer is equal to the rate of supply of the glucose analyte through the AA layer. The degree of permeability (Q_{AS}) of the AA layer to the analyte species (AS) governs the upper limit of analyte concentration for which the sensor has a linear response, along with the activity of the enzyme, as measured by the Michaelis-Menton constant, Km. In the limiting case where both the amount of the enzyme and its activity are sufficiently high, the current can be controlled solely by the membrane permeability to the analyte, (Q_{AS}), and the bulk concentration of the analyte species, $[AS]_B$, as follows (Eq. 6):

$$i = nFQ_{AS}[AS]_B \quad (6)$$

where i, n, and F have the same meaning as stated above. In effect, the steady-state current response is independent of the amount of enzyme activity in the enzyme layer. Such a condition enhances the operational stability and extends the useful shelf-life of the resulting biosensor.

5.1.1. AMPEROMETRIC BASE SENSOR

The amperometric base sensor that is a particular embodiment of the instant invention is fabricated on a substantially planar silicon substrate by means of photolithography in combination with the plasma deposition of

metallic substances. The base sensor may comprise a unit cell containing two catalytic electrodes of identical geometry and area. This configuration allows a differential type of measurement because on only one of these catalytic electrodes is established a biolayer with active enzyme. Such a differential measurement may, in turn, enable the device to measure a current due to the activity of selected bioactive molecules over and above a background level, especially in circumstances where an interfering species may not be readily excluded by a permselective membrane.

Referring now to the accompanying drawings, FIG. 1 illustrates a preferred amperometric glucose sensor unit cell which is repeated in a geometric array several hundred times on a single silicon wafer. Each catalytic indicator electrode, 5 (iridium metal is used in this case), is surrounded by a combined reference and counter electrode, 4 (silver-silver chloride, in particular). The electrodes are each connected to one of three contact pads, 1, by means of an over-passivated signal line, 2. These contact pads serve as the means by which the biosensor is connected to external controlling electronics. The dashed area outlined by 3, represents the passivation layer. The permselective silane layer (functioning as an adhesion promoter and a semipermeable solid film), 6, can be present over the entire structure or, preferably, may be localized on the electrode portions of the unit cell. Over the iridium catalyst are successive overlaid structures: the biolayer or enzyme layer, 7; the AA layer, 8; and the outermost layer, 9, a photoresist cap.

FIG. 2 illustrates the layered structures of one of the pair of indicator electrodes and the reference electrode portion of the preferred differential glucose sensor unit cell. The other member of the pair of indicator electrodes contains no active enzyme in the biolayer, 7. The substrate wafer, 20, is silicon, in this case, with a nonconductive

layer of silicon dioxide, 15, present above it. Patterned titanium metal structures, 10, also serve as conducting signal lines to the contact pads of FIG. 1. The iridium electrocatalyst layer is indicated by 5 in the indicator electrode structure while silver and silver chloride are designated by 4 and 4', respectively, in the reference electrode structure. The polyimide passivation layer is 3 and the permselective silane layer (and adhesion promoter) is 6. Finally, 8 is the analyte attenuation (AA) layer (also, sometimes referred to as the gas permeable membrane elsewhere in this disclosure) and 9 is the photoresist cap.

Although the electrocatalyst is iridium in this particular embodiment, the catalytic metal of the indicator electrode may be made of any of the noble late transition metals. Hence, other metals such as gold, platinum, silver, rhodium, iridium, ruthenium, palladium, or osmium are also suitable. Other elements such as carbon or mercury are also useful. In another embodiment, involving a potentiometric type of electrochemical sensor, a mixed metal oxide alloy such as iridium tantalum oxide may also be used as the metal surface. In yet another possible embodiment, a dioxygen sensor is comprised preferably of a gold indicator electrode. Of these metals, silver, gold, or platinum is preferred as a reference electrode metal. A silver electrode which is subsequently chloridized is most preferred as the reference electrode.

These metals can be deposited by any means known in the art, including the plasma deposition method cited, supra, or by an electroless method which may involve the deposition of a metal onto a previously metallized region when the substrate is dipped into a solution containing a metal salt and a reducing agent. The electroless method proceeds as the reducing agent donates electrons to the conductive (metallized) surface with the concommitant reduction of the metal salt at the conductive surface. The result is a layer

of adsorbed metal. (For additional discussions on electroless methods, see: Wise, E.M. Palladium: Recovery, Properties, and Uses, Academic Press, New York, New York (1988); Wong, K. et al. Plating and Surface Finishing 1988, 75, 70-76; Matsuoka, M. et al. Ibid. 1988, 75, 102-106; and Pearlstein, F. "Electroless Plating," Modern Electroplating, Lowenheim, F.A., Ed., Wiley, New York, New York (1974), Chapter 31.) Such a metal deposition process must yield a structure with good metal to metal adhesion and minimal surface contamination, however, to provide a catalytic metal electrode surface with a high density of active sites. Such a high density of active sites is a property necessary for the efficient redox conversion of an electroactive species such as hydrogen peroxide or dioxygen. Doubtless, equivalent methods of establishing metal layers will be apparent to those skilled in the art.

In addition, the substantially planar substrate need not be a silicon wafer but can be a polished alumina wafer, glass sheet, controlled pore glass, or a planarized plastic liquid crystal polymer. In fact the planar substrate may be derived from any material containing one or more of a variety of elements including, but not limited to, carbon, nitrogen, oxygen, silicon, aluminum, copper, gallium, arsenic, lanthanum, neodymium, strontium, titanium, yttrium, or combinations thereof.

Additionally, the substrate may be coated onto a solid support by a variety of methods well-known in the art including chemical vapor deposition, physical vapor deposition, or spin-coating with materials such as spin glasses, chalcogenides, graphite, silicon dioxide, organic synthetic polymers, and the like. Additional substrates may include gallium arsenide, lanthanum gallate, neodymium gallate, or, less desirably, strontium titanate should the establishment of superconductive materials be deemed desirable.

In one of the initial steps of microfabrication, good metal-substrate adhesion can be promoted by etching the substrate wafer in an argon plasma prior to plasma deposition of titanium metal. The titanium layer serves as the conductive material for the signal lines and also promotes the adhesion of subsequent metal layers onto the substrate surface. The titanium is deposited at a rate of about 2 nm/sec, to a thickness of about 20 to about 500 nm, preferably about 80 nm. This step is followed by plasma deposition of iridium, at a rate of about 0.5 nm/sec to a thickness of about 10 to about 100 nm, preferably to about 20 nm. It is important to exclude dioxygen during metal deposition, since even traces of dioxygen lead to the formation of iridium oxide. Excessive amounts of the oxide provide an inferior sensor surface with a substantially increased capacitance and, therefore, a slower response.

It has been observed that even thin layers of a tenacious residue can reduce the metal surface activity significantly. In this respect, it is important to note that while nonmicrofabricated surfaces can often be reactivated by polishing the electrode with the aid of a slurry of an inert abrasive material, e.g., 0.3 μm particle size alumina powder (See, Sawyer, D.T. and Roberts, J.L. Experimental Electrochemistry for Chemists, Wiley, N.Y. (1974), p. 78), this treatment is incompatible with microfabricated electrode arrays. Hence, in a preferred method for the fabrication of the glucose sensor, it is essential that the polyimide passivation layer (3, in FIGS. 1 and 2) be processed prior to deposition of the catalytic electrode metal. Reversing the order of the processing can lead to the contamination of the catalytic metal surface.

Nevertheless, it has also been discovered that for the purposes of the present invention, passivation of the signal lines is an optional step. To obtain a device which has less topography (i.e., flatter, with fewer ridges), and one which

facilitates the application of layers of materials by wafer spinning with the greatest degree of control, it may even be desirable to discard the polyimide or other passivation layer altogether. This omission is possible, perhaps, because of the observation that titanium, as the metal comprising the signal line, is a poor electrocatalyst for the redox conversion of the electroactive species (e.g., hydrogen peroxide, ascorbate, urate).

5.1.2. ADHESION PROMOTER AND SEMIPERMEABLE SOLID FILM

Another aspect of microfabrication which should be considered when depositing multiple layers onto a planar transducer of this type is the lack of "detailed" rough topography that would promote adhesion between component layers. Frequently, special materials are employed to promote adhesion to the underlying surface. A coupling reagent commonly used for this purpose is γ-aminopropyltrimethoxysilane. The silane compound is usually mixed with a suitable solvent to form a liquid mixture. The liquid mixture can then be applied or established on the wafer or planar sensing device by any number of ways including, but not limited to, spin-coating, dip-coating, spray-coating, and microdispensing. The microdispensing process can be carried out as an automated process in which microspots of material are dispensed at multiple preselected areas of the device (See, further Section 5.4, below). In addition, photolithographic techniques such as "lift-off" or using a photoresist cap may be used to localize and define the geometry of the resulting permselective film (See, Section 6.1.2, infra).

Solvents suitable for use in forming the silane mixtures include aqueous as well as water-miscible organic solvents, and mixtures thereof. Alcoholic water-miscible organic solvents and aqueous mixtures thereof are particularly useful. These solvent mixtures may further

comprise nonionic surfactants, such as polyethylene glycols (PEG) having a molecular weight in the range of about 200 to about 6,000. The addition of these surfactants to the liquid mixtures, at a concentration of about 0.005 to about 0.2 g/dL of the mixture, aids in planarizing the resulting thin films. Also, plasma treatment of the wafer surface prior to the application of the silane reagent can provide a modified surface which promotes a more planar established layer.

Water-immiscible organic solvents may also be used in preparing solutions of the silane compound. Examples of these organic solvents include, but are not limited to, diphenylether, benzene, toluene, methylene chloride, dichloroethane, trichloroethane, tetrachloroethane, chlorobenzene, dichlorobenzene, or mixtures thereof.

When protic solvents or mixtures thereof are used, the water eventually causes hydrolysis of the alkoxy groups to yield organosilicon hydroxides (especially when n = 1) which condense to form poly(organosiloxanes). These hydrolyzed silane reagents are also able to condense with polar groups, such as hydroxyls, which may be present on the substrate surface. When aprotic solvents are used, atmospheric moisture may be sufficient to hydrolyze the alkoxy groups present initially on the silane reagent.

The R' group of the silane compound (where n = 1 or 2) is chosen to be functionally compatible with the additional layers which are subsequently applied. The R' group usually contains a terminal amine group useful for the covalent attachment of an enzyme to the substrate surface (a compound, such as glutaraldehyde, for example, may be used as a linking agent as described by Murakami, T. et al., Analytical Letters 1986, 19, 1973-86; and the article by Yao, T. referred to previously).

In the instant invention, it has been discovered that a film of a silane compound having the formula,

$R'_nSi(OR)_{4-n}$, where n = 0, 1, or 2, which has been heated to at least about 100°C, for a sufficient period of time, usually 5-15 minutes, can dramatically attenuate the transport of interfering electroactive species, ascorbic acid and uric acid, among others, to the electrocatalyst without significantly affecting the current due to the transport of dioxygen and hydrogen peroxide. In a preferred embodiment of the present invention, the R' fragment of the silane is a hydrocarbon radical comprised of 3-12 carbon atoms and R is a lower alkyl radical comprised of 1-4 carbons. In addition, the R' hydrocarbon fragment may further comprise at least one heteroatom such as an oxygen, nitrogen, phosphorus, or sulfur. Further, functional groups which represent stable combination of these heteroatoms, such as isocyanato, cyanato, phosphate, and the like may also be present. It may even be desirable in certain instances to employ an organosilane reagent in which the hydrocarbon fragment R' further comprises a suitable leaving group, preferably at the terminus of the hydrocarbon fragment. One example of such a silane reagent is 3-chloropropyltrimethoxysilane. In this manner, nucleophilic moieties may be covalently bound to the silane layer by displacement of the potential leaving group.

The lower alkyl radical, R, may be a methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, tertiary butyl group, or mixtures thereof. Routine experimentation will determine which groups are best suited to the particular manufacturing conditions being employed. Such factors as volatility, boiling point, and viscosity properties may be important considerations. The ease by which the alkoxy groups are hydrolyzed may also be dispositive. Also, because the -OR groups are substantially hydrolyzed in an aqueous environment, silane reagents in which R is also a hydrogen radical, is within the scope of the present invention.

Indeed, an important aspect of the present invention is the discovery and recognition that certain classes of silane reagents can be formulated into a convenient medium, established onto a substantially planar surface, and subsequently treated under controlled conditions to provide a layer or coating with permselective properties. It should be pointed out that prior to these observations, silane reagents were employed as mere adhesion promoters, on the one hand, or to establish impermeable glasses, at the other extreme.

Hence, freshly prepared alcoholic solutions of silicon hydroxides can be spun onto a wafer and heated to an intermediate degree such that the dehydration reaction which accompanies such heating produces a material having semipermeable properties. Although, the -OR groups of the silane reagent are preferably hydrolyzed (and later dehydrated), it should be pointed out that such hydrolysis is not always necessary. The thermal conversion of a tetraalkoxysilane to an intermediate form of silicon dioxide can be accompanied by the evolution of an ether compound.

It has been discovered further that replacing one or two of the alkoxy or hydroxy groups of tetrasubstituted siloxane with a group which is not readily hydrolyzed, such as a hydrocarbon moiety bonded directly to silicon, renders the resulting silane layers "more porous" than their "glassy" counterparts. Thus, for a given thickness, a layer derived from a silane of the formula $Si(OR)_4$ is less permeable than one obtained from a reagent of the formula $R'Si(OR)_3$. The increased permeability of the latter is, perhaps, best explained by the inferior ability of the $R'Si(OR)_3$ precursor to establish a network of oxo-bridged silicon atoms.

For optimum performance, then, the thickness and composition of the silane layer must be controlled. Such control is achieved by carefully selecting the identity of the silane reagent used, adjusting its concentration in the solvent mixture, and determining the proper rotation speed if

the solution of the silane is deposited onto the wafer by spin-coating. Numerous silane compounds such as 3-aminopropyltriethoxysilane, N-(2-aminoethyl)-3-aminopropyltriethoxysilane, 3-aminopropyltrimethoxysilane, N-(2-aminoethyl)-3-aminopropyltrimethoxysilane, 3-isocyanatopropyltriethoxysilane, 10-aminodecyltrimethoxysilane, 11-aminoundecyltrimethoxysilane, 2-[p-(N-(2-aminoethyl)aminomethyl)phenyl]ethyl-trimethoxysilane, n-propyltrimethoxysilane, phenyltrimethoxysilane, diethylphosphatoethyltriethoxysilane, or N,N-bis(2-hydroxyethyl)aminopropyltriethoxysilane, 3-chloropropyltriethoxysilane, are commercially available and may be processed in this manner to yield a semipermeable solid film which promotes adhesion of subsequent layers of other materials and yet is able to act as a small-molecule-selective membrane. As stated earlier, other materials used as preceramic or precursors to dielectric layers may also be used under proper conditions. Silicafilm products available from Emulsitone Company (Whippany, New Jersey 07981) may be utilized. Examples of other silanes include tetrahydroxyorthosilicates (silicic acid) or tetraalkylorthosilicates such as tetramethyl, tetraethyl, tetrapropyl, tetrabutyl orthosilicates, or their mixtures. However, the preferred silane compound is N-(2-aminoethyl)-3-aminopropyl(trimethoxy)silane. The resultant sensor is easy to manufacture, has a very fast response time to changing hydrogen peroxide concentrations, and is substantially free of the signals resulting from interfering electroactive species.

As mentioned previously, the degree of permeability of the permselective silane membrane is due not only to the nature of the silane reagent but is also largely dependent on its thickness. A useful range of thickness lies in the range of about 1 to about 1000 nm, preferably between about 2 to about 20 nm. However, where one desires to substantially